

Osteoarthritis and Cartilage (2004) 12, 917–923

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doi:10.1016/j.joca.2004.08.003

Osteoarthritis and Cartilage

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Characterization of mature vs aged rabbit articular cartilage: analysis of cell density, apoptosis-related gene expression and mechanisms controlling chondrocyte apoptosis¹

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Summary

Objective: The prevalence of osteoarthritis (OA) is increased in aged individuals and a direct correlation between chondrocyte apoptosis and cartilage degradation secondary to OA has been demonstrated. To address the question of whether age predisposes articular cartilage to apoptosis, the objective of the present study was to characterize and compare in aged and mature non-OA rabbit articular cartilage, cell density and expression levels of specific genes associated with apoptosis. Mechanistic studies on the inhibition of induced apoptosis were also carried out.

Methods: Grade I (non-OA) femoral condyles and tibial plateaus from mature and aged rabbits were taken for assessment of viable cell density (VCD) and mRNA (reverse transcription-polymerase chain reaction) expression levels of the pro-apoptotic genes, Fas, Fas ligand (FasL), caspase-8, inducible nitric oxide synthase (iNOS) and p53. *In vitro* insulin-like growth factor (IGF-1)-mediated inhibition of nitric oxide (NO)-induced apoptosis was also examined using sodium nitroprusside (SNP) as NO donor.

Results: VCD was decreased 50–70% in aged articular cartilage relative to mature cartilage. mRNA expression levels of Fas, FasL, caspase-8 and p53 were higher in aged cartilage than in mature cartilage. iNOS expression was unchanged. IGF-1-mediated inhibition of NO-induced apoptosis was dose-dependent and reversed with addition of phosphatidylinositol-3 kinase inhibitor.

Conclusions: This controlled animal model study demonstrates that age predisposes articular cartilage to changes in VCD and expression levels of specific pro-apoptotic genes. It is significant that these findings were demonstrated on cartilage that showed no prior signs of OA; it is also possible that such changes are a prelude to the age-related development of OA.

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Key words: Apoptosis, Articular cartilage, Aging, Chondrocytes.

Introduction

The prevalence of osteoarthritis (OA) is profoundly increased in aged individuals, and it appears that aged cartilage may incur changes which directly predispose it to the development of OA. Indeed, age is the most consistent epidemiological feature of OA. However, the genetics and molecular biology linking age to this debilitating disease have yet to be elucidated. Moreover, the specific alterations in gene expression in normal aging cartilage are largely unknown.

In humans, substantial decreases in chondrocyte cell density occur in articular cartilage during both aging and the development of OA^{1–4}. Recent studies suggest that this decline in cell density in aging and osteoarthritic human cartilage occurs primarily in the superficial cell layer, without significant changes in the middle or deep zones⁴. However, age-related human cartilage cellularity studies have been

limited by a lack of complete accounting of patient lifestyles, genetic backgrounds, and weight-bearing areas in articular cartilage. Some of these limitations are addressed in the present animal model study using New Zealand white (NZW) rabbits.

Cell loss in aged or OA cartilage was initially thought to occur by necrosis⁵. However, more recent reports suggest that cell death occurs primarily via apoptosis or apoptosis-like programmed cell death^{6–9}. *In situ* TUNEL and electron microscopy studies on normal and osteoarthritic human cartilage have shown increased chondrocyte apoptosis in OA tissue^{6,7}. Apoptosis has also been positively correlated with the severity of cartilage destruction and with matrix depletion in human specimens⁸. Factors that can induce chondrocyte apoptosis include nitric oxide (NO) and NO donors such as sodium nitroprusside^{10–13} (SNP), Fas–Fas ligand (FasL)¹⁴, hypertrophy, staurosporine, loss of extracellular matrix (ECM) and the resulting lack of cell adhesion to ECM^{6,7,13,14}. NO and the Fas–FasL molecules have been shown to be present in OA joints and are thought to be key contributors to chondrocyte cell loss, defective cartilage homeostasis and joint destruction¹⁴. OA chondrocytes express inducible nitric oxide synthase (iNOS) and produce NO spontaneously, unlike chondrocytes from normal non-OA cartilage¹⁵. Moreover, elevated levels of NO and FasL

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Received 17 March 2004; revision accepted 11 August 2004.

have been found in the serum and synovial fluid of patients with OA^{16–18}. NO also suppresses cartilage matrix production and activates matrix metalloproteinases (MMPs)^{19,20}. Functionally, recent *in vitro* studies have shown that NO, using SNP as donor, promotes chondrocyte dedifferentiation and activation of the MAP kinase members ERK1/2 and p38.

In chondrocytes, as in other cell types, the interaction of the tumor necrosis factor (TNF) receptor family member Fas with FasL leads to the proteolytic activation of caspase-8, an upstream protease enzyme which plays a central role in apoptosis^{14,21,22}. Activation of upstream caspases (e.g. -8, -9) leads to the proteolytic activation of downstream or effector caspases (e.g. caspase-3, -6, and -7), resulting in cell destruction by apoptosis via the cleavage of structural and regulatory proteins. Finally, p53, a gene previously reported to be associated with apoptosis in numerous tissues has also been shown to correlate with apoptosis in articular cartilage and the degree of ECM degradation²³.

In joints affected by OA or aging, a decreased sensitivity to critical anabolic factors, such as insulin-like growth factor (IGF-1), might also exist. A key function of IGF-1 is as a potent inhibitor of apoptosis in many cell types, including chondrocytes²⁴. Such inhibition may occur in several ways. Recent studies on cultured chondrocytes from 2-week-old rabbits have shown that IGF-1 inhibits NO-induced chondrocyte apoptosis via phosphatidylinositol-3 kinase (PI3 kinase)²⁵. Although it is unclear whether this occurs in mature or aged rabbit chondrocytes and by what mechanisms downstream of PI3 kinase, these findings improve our understanding of this complex pathway.

With data on aged non-OA cartilage being very limited, the purpose of the present study was first, in a controlled animal model, to define changes in chondrocyte cell viability, number, and cell density in articular cartilage as a function of age and second, to identify the mediators behind these changes by comparing the expression of the apoptosis-related genes Fas, FasL, caspase-8, iNOS and p53 in grade I (non-OA) knee articular cartilage from mature and aged rabbits. The effectiveness of IGF-1 on chondrocyte survival was subsequently evaluated during NO-induced apoptosis (using SNP as NO donor) of mature rabbit chondrocytes *in vitro* as well as by examining downstream inhibitors in the PI3 kinase and MEK/ERK1/2 kinase pathways.

Materials and methods

CELL VIABILITY AND CELL DENSITY

For cell viability and density analysis, only joints receiving a macroscopic grade I by the Outerbridge classification (intact femoral and tibial surfaces) after india ink staining were used. Each articular cartilage layer (superficial, middle and deep) was analyzed simultaneously using a fluorescent *in situ* double-staining protocol and laser scanning confocal microscopy to maximize accuracy²⁶. Full-thickness cartilage from mature (9–12 months with closed epiphyses) and aged (4–5 years) rabbits ($n = 6$ each) removed from subchondral bone was sectioned into 0.5–1.0 mm coronal slices and then placed into a solution containing 2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), which stains viable cells green, and propidium iodide (PI), which stains nonviable cells red. Specimens in solution were placed in a 37°C CO₂/O₂ incubator for 30–45 min and then removed for microscopic

examination. Specimens were viewed with a Zeiss LSM 510 laser confocal scanning microscope equipped with krypton and argon lasers (Carl Zeiss, Inc., Thornwood, NY). Images were obtained at multiple random locations within the cartilage slice using Zeiss laser scanning systems LSM 510 software. Viable and nonviable cells were quantitated by computer software (Image-Pro Plus 4.1, Media Cybernetics, Inc., Silver Springs, MD). The confocal laser microscope was programmed to image at multiple, defined focal depths. Images were then reconstructed utilizing the Image Tool Analysis Program (NIH Image 1.6, National Institutes of Health, Bethesda, MD) producing a three-dimensional image cube with an optical section dimension of 0.7 mm × 0.7 mm and a reconstructed focal depth of 0.025 mm (volume = 0.7 mm × 0.7 mm × 0.025 mm = .01225 mm³). The viable cell density (VCD; viable cells per unit volume) was determined by calculating the number of viable cells within this known volume and expressed as cells per mm³.

GENE EXPRESSION BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Grade I ($n = 6$ each, mature and aged) articular cartilage from the knees of NZW rabbits was harvested after sacrifice with ketamine and beuthanasia solution injections. Following careful dissection of the articular surfaces, the articular cartilage was pulverized in liquid nitrogen and RNA extracted using an acid-guanidinium-thiocyanate–phenol extraction procedure²⁷. Starting with 1 µg of total RNA, first-strand cDNA was synthesized using oligo(dT)₁₅ primers. Based on published sequences (BLAST), polymerase chain reaction (PCR) primer sets specific to selected coding regions of Fas, FasL, caspase-8, p53, iNOS and GAPDH were constructed for each gene (Table I). Amplifications were allowed to proceed through a cycle range of 24 to 38 cycles in order to establish the linear range of amplification for each gene. Based on this, 30 cycles were used for GAPDH and 34 cycles were used for all other genes. Amplifications were carried out simultaneously for all genes to increase accuracy of comparisons. PCR products were subjected to agarose gel electrophoresis and ethidium bromide visualization. NIH Image analysis software (version

Table I
PCR primer sequences (BLAST) used for specific genes

Gene	Sequence	PCR product size (bp)
GAPDH	5'-TCACCATCTTCCAGGAGCGA-3' 5'-CACAATGCCGAAGTGGTCGT-3'	293
Fas	5'-CAAACGAGCAACACCAAATGC-3' 5'-CCGCAAGAGCACAAAGATTAGC-3'	395
FasL	5'-GAAATGGGAAGACACATATGGA- ACTGC-3' 5'-CATATCTGGCCAGTAGTGCACT- AATTC-3'	274
Caspase-8	5'-ACTGCTTCATCTGCTGTATC-3' 5'-CAGGCTCTGGCAAAGTGACT-3'	305
iNOS	5'-CATCCCAATACGAGTGGTT-3' 5'-GGTCCATGATGGTCACATTC-3'	262
p53	5'-GACGTTGCCAACTGGCTCAA-3' 5'-CGTCATGTGCTGAGACTTCT-3'	301

1.61, National Institutes of Health, Bethesda, MD) was used to quantitate the band densities corresponding to the PCR products. The NIH software measures relative mean density over a fixed gray scale range after correction for background. Expression for each gene is shown as a ratio of GAPDH.

MONOLAYER CHONDROCYTE CELL CULTURE

Grade I ($n = 8$) femoral and tibial articular knee cartilage from mature NZW rabbits was aseptically dissected, sliced and enzymatically dissociated for 4–6 h in 0.2% collagenase type II (381 units/mg solid; Sigma) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 50 μ g/ml streptomycin and 50 units/ml penicillin. Suspended chondrocytes and debris were then sterile filtered and plated on culture dishes at a density of $\sim 5 \times 10^4$ cells/cm² in DMEM supplemented with 5% (v/v) fetal bovine serum (FBS), streptomycin and penicillin. Media was changed every 2 days and experiments performed when cell confluence reached approximately 90%. Cells were used for experiments only as primary cells or after first passage.

SNP-INDUCED APOPTOSIS AND IGF-1-RELATED REVERSAL

The NO donor, SNP, was used to induce rabbit chondrocyte apoptosis in cell culture. In our studies, SNP treatment for approximately 16 h was employed at increasing concentrations to generate a dose–response curve. Cell signaling pathway inhibitors, including the MAP kinase-kinase (MEK) inhibitor UO126 (Calbiochem) and PI3 kinase inhibitor wortmannin (200 nM, Calbiochem) or LY294002 (5 nM, Calbiochem) were dissolved in dimethyl sulfoxide (DMSO) then resuspended in a 1:1 mixture of DMSO:serum-free DMEM for use at the

above concentrations in cell culture experiments. IGF-1 (Sigma) was used at 100 ng/ml unless otherwise stated. Inhibitors were added after a 24–36 h incubation in DMEM/0.5% FBS. Following a 20–30 min incubation with the inhibitors, IGF-1 was added and experimental incubation begun. Cells were then released from the culture dish by gentle treatment with trypsin and washed twice in phosphate-buffered saline, pH 7.4. Cells were then placed into a calcium-containing HEPES buffer and fluorescein isothiocyanate-labeled annexin V and PI were added for determination of apoptosis. Apoptosis was assessed by FACS detection of annexin V cell staining and the number of these cells is reported here as percent apoptosis.

STATISTICAL ANALYSIS

All data are expressed as mean \pm standard deviation. For semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) studies, aged vs mature comparisons were achieved using unpaired Student's *t* test where statistical significance was set at $P < 0.05$. For cell viability and VCD studies, statistical analysis was achieved using Mann–Whitney's *U* test with statistical significance also set at $P < 0.05$.

Results

MACROSCOPIC JOINT GRADING FOR CELL VIABILITY AND CELL DENSITY

In mature rabbits, all cartilage was grade I except for two medial tibial plateaus (MTPs) that were grade II. In aged rabbits, 12 out of 12 lateral femoral condyles, 11 of 12 lateral tibial plateaus, 9 of 12 medial femoral condyles and 9 of 12 MTPs were grade I. Examples of grade I articular cartilage are shown in Fig. 1. The remaining specimens

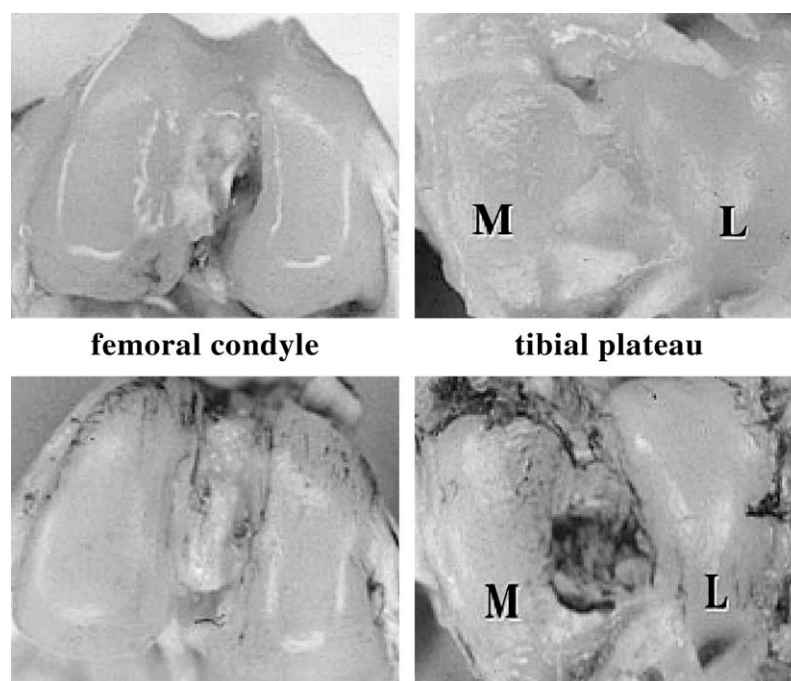


Fig. 1. Example of grade I articular cartilage on a rabbit femoral condyle and tibial plateau. Top panels are photographs of untreated joints and bottom panels are after india ink staining. M: medial; L: lateral.

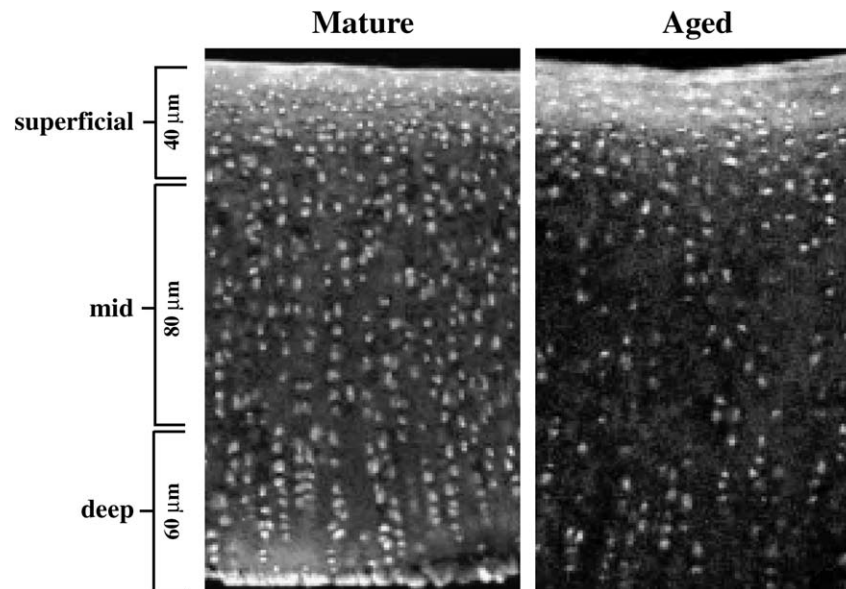


Fig. 2. Example of confocal microscopy following staining for viable chondrocytes in the superficial, mid and deep zones of mature and aged rabbit tibial plateau articular cartilage. Note: brightly white-stained chondrocytes indicate viable cells.

were early grade II and were not used in the determination of cell viability or density studies.

CELL VIABILITY AND VCD

Figure 2 shows an example of confocal microscopy following double staining for viable (BCECF-AM) and nonviable (PI) chondrocytes in the superficial, mid and deep zones of mature and aged rabbit articular cartilage. Results of NIH Image analysis of VCD following confocal microscopy are shown in Table II and Fig. 3. In both the femoral condyle and tibial plateau, aged cartilage showed significant ($P < 0.05$) decreases in VCD within each layer of articular cartilage: 57% in the superficial zone, 52% in the middle zone and 59% in the deep zone for tibial plateau cartilage and 42%, 26% and 31%, respectively, for the femoral condyle.

SEMI-QUANTITATIVE RT-PCR ANALYSIS OF APOPTOSIS-RELATED GENES

RT-PCR followed by agarose gel electrophoresis of GAPDH, Fas, FasL, caspase-8, iNOS and p53 was performed on six specimens each of mature and aged articular cartilage. A representative result for one set each of mature and aged cartilage is shown in Fig. 4. The results of semi-quantitative analysis (NIH Image software) of the RT-PCR experiments are illustrated in Fig. 5. This figure shows that Fas, FasL, caspase-8 and p53 were all

expressed at statistically higher levels ($P < 0.05$) in aged rabbit articular cartilage than in mature cartilage: respectively, 1.02 ± 0.37 vs 0.3 ± 0.25 for Fas; 0.48 ± 0.04 vs 0.21 ± 0.06 for FasL; 0.77 ± 0.33 vs 0.20 ± 0.06 for caspase-8; 1.29 ± 0.55 vs 0.63 ± 0.32 for p53. No statistically significant difference in iNOS expression was found between mature and aged cartilage.

IGF-1-MEDIATED CHONDROCYTE SURVIVAL DURING SNP-INDUCED DEATH

NO-induced apoptosis of chondrocytes occurred in a dose-dependent manner at SNP concentrations between 0.25 and 1.00 mM (Fig. 6). At 0.500 mM SNP, 18.5% cells were strictly apoptotic and IGF-1 inhibited apoptosis by $52 \pm 11\%$ ($P < 0.05$; Fig. 6). IGF-1-mediated apoptosis inhibition was reversed with the addition of the PI3 kinase inhibitor, wortmannin. However, inhibition of the MEK/ERK kinase pathway did not significantly alter IGF-1-induced apoptosis inhibition (Fig. 7). Isolated inhibition of either PI3 kinase or MEK-ERK did not significantly induce chondrocyte apoptosis over that of baseline controls.

Discussion

Chondrocyte cell loss in developing OA in humans is an established phenomenon that leads to alterations in cartilage matrix production and the ability to withstand mechanical loads. Concurrently, chondrocyte signaling, gene expression and phenotype are altered, leading to apoptosis in a cyclic pattern of eventual cartilage degradation and worsening OA. In this study of aging non-OA cartilage in a rabbit model, substantial decreases in cell density were observed in each layer of aged articular cartilage. We also found that aged chondrocytes expressed higher levels of specific pro-apoptotic genes and that IGF-1 significantly protects mature chondrocytes from NO-induced apoptosis in a PI3-kinase-dependent manner. This is the first report we are aware of which characterizes

Table II
VCD of rabbit articular cartilage (10^4 cells/mm³)

		Superficial	Middle	Deep
Mature	Femoral condyle	8.3 ± 2.3	11.8 ± 2.0	10.0 ± 1.3
	Tibial plateau	8.8 ± 3.0	6.0 ± 2.3	6.8 ± 1.0
Aged	Femoral condyle	4.8 ± 2.4	8.7 ± 3.0	6.9 ± 2.0
	Tibial plateau	3.8 ± 1.7	2.9 ± 1.1	2.8 ± 0.7

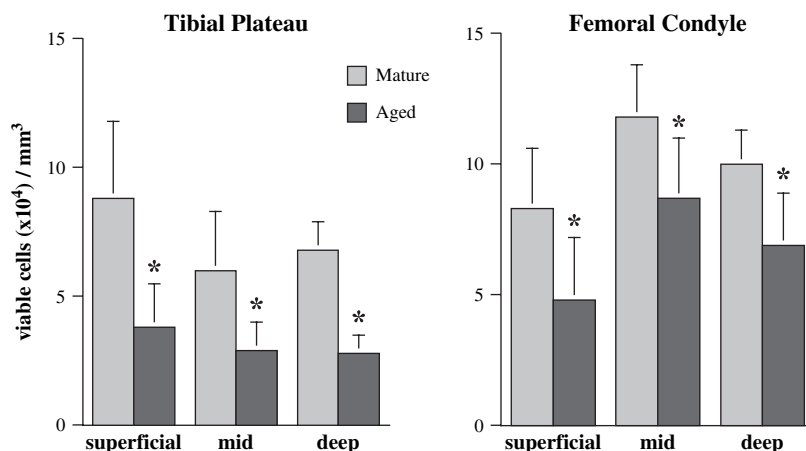


Fig. 3. VCD in the superficial, mid and deep zones of mature and aged ($n = 6$, each) rabbit tibial plateau and femoral condyle articular cartilage: * = $P < 0.05$, aged vs mature.

changes in both cartilage cell viability/density and apoptotic gene expression as a function of age in non-osteoarthritic articular cartilage.

External signals which alter aged chondrocyte gene expression and increased apoptosis are pivotal to our understanding of how the transition from normal to aged to OA cartilage occurs. While molecular mechanisms which predispose chondrocytes *in vivo* to undergo apoptosis have not been fully elucidated, factors which have been shown to affect the rate of apoptosis during degenerative joint disease include increased cartilage loads⁹, cartilage lipid oxidation²⁸, matrix depletion⁸ and NO^{14,15}. Several human and animal cartilage studies have demonstrated that during OA there are increases in both chondrocyte apoptosis and in the expression of the apoptosis-related genes Fas, Bcl-2, FasL, iNOS, p53 and Bax^{6,7,14,15}. There also appears to be increased levels of proteolytic caspases, specifically caspase-8 and -9, in human OA cartilage^{15,16}.

In the present study, relative to mature cartilage, we found significant increases in Fas, FasL, caspase-8 and p53 gene expression in aged cartilage. While Fas expression in human OA samples and its increased expression in aged non-OA cartilage observed in this study may coincide, it is unclear whether this represents a continuum from progressive age-related changes toward OA. Furthermore, while we observed Fas and FasL expression to be increased in non-OA aged rabbit cartilage, studies in human OA samples conflict regarding their roles¹⁴. Generally, downstream caspases implement cell destruction during apoptosis, such that increased expression of caspase-8 may represent a correlation with general stimuli inducing apoptosis or specifically with the expression of Fas, an upstream regulator. p53, an important regulator of apoptosis in numerous tissues, also showed increased expression in aged non-OA cartilage. Chondrocyte fate in aging and in OA, therefore, appears to depend on the

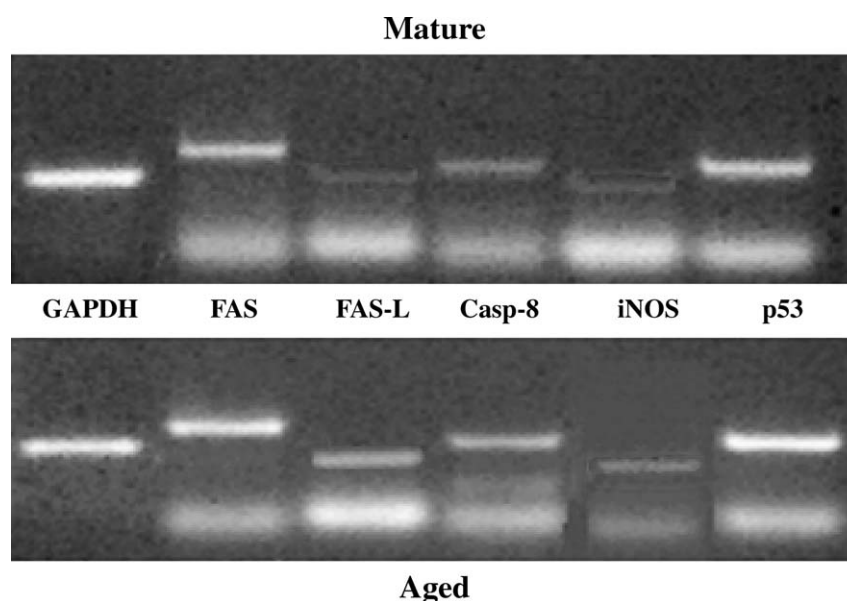


Fig. 4. Agarose gel electrophoresis following RT-PCR of RNA extracted from representative samples of mature and aged rabbit articular cartilage.

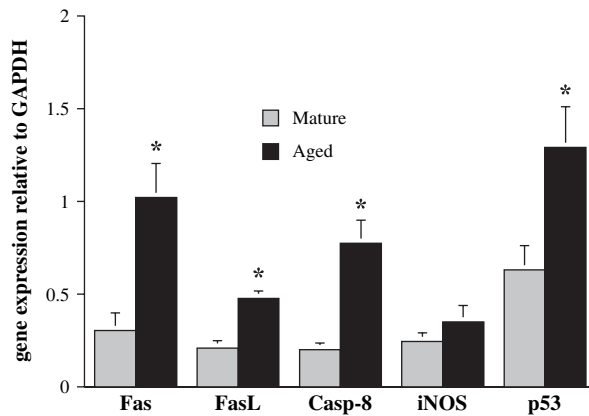


Fig. 5. Relative expression of apoptosis-related genes in mature and aged rabbit articular cartilage as determined by NIH Image analysis following RT-PCR and agarose gel electrophoresis: * = $P < 0.05$, aged vs mature; $n = 6$, each.

integration and cellular interpretation of these signals and their relative abundance in cartilage. This complex interplay of molecular signaling occurs between the cell membrane and its nucleus to alter gene expression, determining cell differentiation, status and fate.

Mechanistically, *in vitro* studies on rabbit chondrocytes using SNP as an NO donor have shown that NO promotes chondrocyte dedifferentiation, activation of extracellular-signal regulated kinase ERK1/2, and the activation of p38 stress kinase^{10–12}. Chondrocyte dedifferentiation is associated with increased ERK1/2 activation, while NO-induced apoptosis appears to be p38-dependent and leads to NF- κ B-dependent p53 gene upregulation^{10–12}. In the present study we found a dose-dependent induction of apoptosis in mature chondrocytes treated with SNP. This shows that in mature rabbit chondrocytes, NO, via SNP, induces apoptosis but does not elucidate the pathway of cell death.

Inhibitors of chondrocyte apoptosis and their mechanisms of action are not well understood. IGF-1, a major anabolic factor in cartilage, is a single polypeptide homologous to proinsulin which primarily activates the Ras-Raf-MEK1/2-ERK1/2 MAPK pathway (a key component in MMP induction by cytokines and apoptosis^{29,30}) and

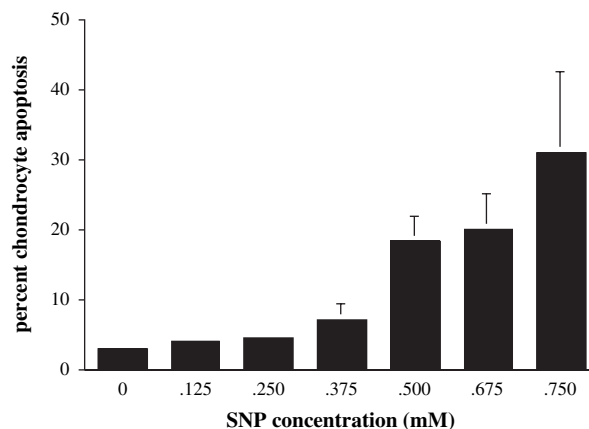


Fig. 6. Dose-dependent apoptotic response of mature rabbit articular chondrocytes to nitroprusside (SNP); $n = 8$.

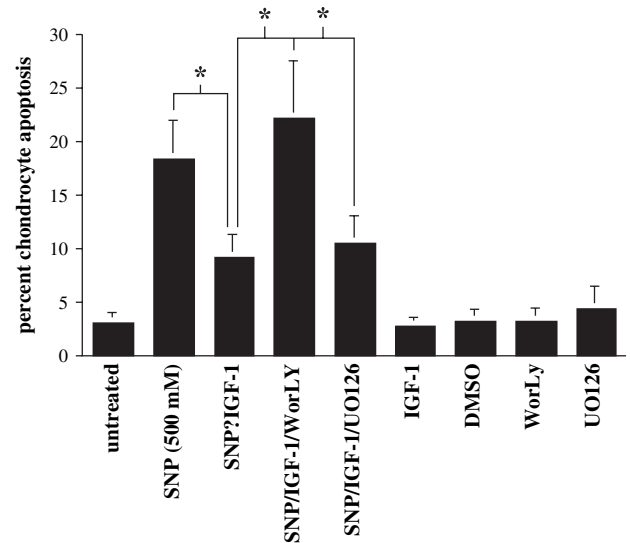


Fig. 7. The effect of PI3 kinase inhibitor and MEK-MAPK kinase inhibitors on IGF-1 inhibition of SNP-induced apoptosis: * = $P < 0.05$, $n = 8$. Note that the PI3 kinase inhibitor reversed IGF-1 inhibition but MEK-MAPK inhibitor had no effect.

the PI3 kinase signaling cascade. In cultured rabbit chondrocytes treated with SNP, ERK1/2 is activated and leads to cell dedifferentiation but not apoptosis; in human chondrocytes, MEK1/2 inhibition (by UO126) leads to caspase-3 activation and apoptosis³¹, such that MEK may play a pro- or anti-apoptotic role. Our data show that in mature rabbit chondrocytes, NO-induced apoptosis is inhibited by IGF-1. By using specific PI3 kinase and MEK inhibitors, we were also able to demonstrate that this inhibition of apoptosis by IGF-1 occurred in a PI3-kinase-dependent, but not MEK-ERK-dependent fashion, implying that the PI3 kinase is likely a downstream target of IGF-1 in this experiment. A study by Oh and Chun in 2-week-old chondrocytes showed similar findings, in that IGF-1 inhibited NO-induced dedifferentiation and apoptosis of rabbit chondrocytes through the activation of PI3 kinase and the subsequent blockage of p38 kinase and ERK1/2²⁵. Their study also suggests that PI3 kinase and the downstream survival signal Akt are inhibited during articular cartilage NO production, an apparent prerequisite for cell apoptosis and dedifferentiation. In our present study, however, inhibition of PI3 kinase using wortmannin or LY294002 did not increase apoptosis over untreated controls. Thus, although inhibiting PI3 kinase and Akt may be a prerequisite for chondrocyte apoptosis, their isolated inhibition did not directly induce apoptosis, implying that another stimulus is required.

Although the changes characteristic of aged cartilage and the complex pathways which signal these alterations are still not fully understood, it appears that an imbalance exists in the aged or osteoarthritic joint in which chondrocytes become more sensitive to excess catabolic stimuli while simultaneously becoming less sensitive to critical anabolic factors like IGF-1. Such an imbalance may play a pivotal role in the transition of aged cartilage toward increased pro-apoptotic gene expression and cellular loss as seen in the present study. A shift toward catabolic stimuli in the aged joint likely leads to failure of repair mechanisms, altered gene expression and eventual cell death by apoptosis. We believe that an upregulation of specific pro-apoptotic genes

in aged non-OA chondrocytes leads to chondrocyte apoptosis and predisposes the aged articular cartilage to cell loss and subsequent events that result in progressive degeneration of the aging articular cartilage.

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